1-1-1995

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Recommended Citation

Schnell, S., & King, G. (1995). Stability of methane oxidation capacity to variations in methane and nutrient concentrations. *FEMS Microbiology Ecology, 17* (4), 285-294. https://doi.org/10.1016/0168-6496(95)00034-8

ELSEVIER

FEMS Microbiology Ecology 17 (1995) 285–294

FEMS MICROBIOLOGY ECOLOGY

Stability of methane oxidation capacity to variations in methane and nutrient concentrations

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Received 13 December 1994; revised 9 May 1995; accepted 15 May 1995

Abstract

Uptake rate constants for atmospheric methane consumption by the 4–6 cm depth interval of a forest soil did not change during 4 months incubation in vitro, even though atmospheric methane concentrations were significantly higher than in situ concentrations. Uptake rate constants were also unaffected by continous incubation at a constant methane concentration of 17 ppm for 2 weeks and 170 ppm for 3 weeks. Uptake rates during incubation with 1000 ppm methane increased 176-fold when assayed with 1000 ppm methane and 5.5-fold when assayed with 1.7 ppm methane. These enhancements were lost after subsequent incubation with atmospheric methane. The ratio of methane oxidized to carbon dioxide produced varied from 49–53% at methane concentrations up to 170 ppm. Incorporation of ^{14}C-methane into phospholipids was 0.35% and 0.22% at atmospheric and 170 ppm methane concentrations, respectively, suggesting that patterns of assimilation were independent of methane concentrations. Addition of several carbon substrates (glucose, starch, yeast extract, methanol, ethanol, formate, acetate, malate, or lactate) to soils incubated at 1.7 or 100 ppm methane did not stimulate methane oxidation. Addition of copper, nitrate or a mineral medium also did not affect methane oxidation. However, incubations with 0.2 or 2% oxygen resulted in lower activity than with ambient air. The methane-consuming capacity of soil decreased exponentially with time when starved for methane by continuous incubation with air containing < 0.03 ppm methane. After 6.3 days of starvation, the soil lost 50% of its original activity; activity was not recoverable after further incubation with atmospheric methane. Methane uptake by soil was rapidly inhibited by the addition of antibacterial antibiotics (streptomycin, chloro-tetracycline, chloramphenicol, ampicillin) as well as by the eukaryotic antibiotic, cycloheximide. Culture suspensions of *Methylosinus trichosporium* OB3b showed a similar sensitivity to both types of antibiotics. Cell suspensions of *Methylosinus trichosporium* OB3b and *Methylobacter albus* consumed atmospheric methane, but consumption rates decreased continuously over a period of 15 days. In contrast, methane consumption by soil incubated under the same conditions was temporally stable. However, cell suspensions of both cultures showed higher consumption rates for atmospheric methane when sprayed on sand relative to incubations in liquid media.

Keywords: Atmospheric methane; Soil methane consumption; Various methane concentrations; *Methylosinus trichosporium* OB3b; *Methylobacter albus*

1. Introduction

Atmospheric methane consumption has been reported for various temperate and tropical forest,

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SSDI 0168-6496(95)00034-8

grassland and tundra soils [1,4,13,18,25–27]. The role of terrestrial environments as the only net methane sink has been recognized as an important component of global methane dynamics. Controls of atmospheric methane consumption are currently the focus of intensive study, and some regulatory factors have been identified. Soil moisture strongly affects the capacity for methane consumption by determining the extent of diffusion between the soil gas phase and the atmosphere [1,6,7,27]. Soil nitrogen, and ammonium in particular, can effectively inhibit methane oxidation. Ammonium competes with methane at the level of methane monooxygenase; it is first oxidized to hydroxylamine and then to nitrite, both of which are toxic intracellular products [10,11,17,20,28]. Ammonium appears to inhibit methane consumption in the surface layer of soils [24], possibly accounting for the typical sub-surface (4–8 cm) maximum of methane-consuming activity. Ammonium-oxidizing bacteria have been suggested as the organisms responsible for atmospheric methane consumption [19,25]. However, the location of maximum potential nitrification rates at the soil surface where concentrations of ammonium and nitrate are highest [24] is inconsistent with significant methane consumption.

Kinetic studies of methane oxidation by soil have shown a low V_{max} and high affinity activity associated with atmospheric methane consumption, but a high V_{max} and low affinity activity after preincubation of soil with high methane mixing ratios (20%; [4]). The latter activities are consistent with the characteristics of known cultures of methanotrophs and methane-oxidizing nitrifiers. Bacteria with affinities for methane comparable to those of soil remain uncultivated. Whether or not methane-oxidizing bacteria can ever grow at ambient methane concentrations is also unclear. Conrad [5] concluded from the kinetic characteristics of existing cultures that ambient methane mixing ratios are probably too low to allow growth. However, the ubiquity of methane consumption by diverse soils suggests that at least some methanotrophs are capable of maintenance and probably slow growth.

We have characterized the response of atmospheric methane consumption to possible co-substrates and a variety of potentially limiting inorganic nutrients. The response to methane starvation and to elevated methane levels was examined. Two strains of methanotrophs (*Methylobacter albus*, group I and *Methylosinus trichosporium* OB3b group II) were examined for their ability to oxidize atmospheric methane and compared to those of soil.

2. Materials and methods

Forest soil (pH 4) was collected with acrylic tubes from a mixed hardwood-conifer forest adjacent to the Darling Marine Center, Walpole, ME. Detailed site descriptions and sample collections are given in King and Adamsen [14]. For most experiments, soil was collected exclusively from the most active depth interval (4–6 cm). Soil was sieved (5 mm) to remove stones and roots and pre-dried at ambient laboratory temperature if necessary to a water content of approximately 25%.

For the enrichment experiments with soil cores, six plexiglas core tubes (6.4 cm diameter, 30 cm length) were sealed at the bottom with butyl rubber stoppers, and filled to a height of 16 cm with well-mixed soil from the depth interval of maximum methanotrophic activity (4–6 cm). The cores were placed in a plastic incubator to maintain a constant moisture content, and were supplied continuously with ambient air or air/methane mixtures. Methane uptake was followed frequently by temporarily sealing the cores and measuring headspace methane concentrations. Cores were incubated initially with ambient methane concentration (1.7 ppm); after 4 months, methane concentrations were increased stepwise to 17 ppm (for 2 weeks), 170 ppm (for 3 weeks), and finally 1000 ppm (for 5 weeks). Water content of the cores were followed by determining core weights and adding water at the soil surface as necessary. Depth profiles of methane in the cores were obtained with stainless-steel needles fitted with plastic luer-lok syringes. Methane consumption rates at different depths were measured by sectioning three of the soil cores and placing 5 g soil into 120-ml glass jars that were sealed with rubber stoppers. Methane concentrations in the jar headspaces were followed at intervals.

Different substrates were added separately as aqueous solutions to soil samples (1 ml to 10 g soil) that were mixed gently and placed into sealed 120-ml

jars. Controls were supplemented with equal amounts of deionized water. Soils were incubated with 100 ppm methane and organic substrates (10 μmol glucose, 2 mg starch, 2 mg yeast extract, 10 μmol methanol, 20 μmol ethanol, 20 μmol formate, 20 μmol acetate, 10 μmol β-hydroxybutyrate, 10 μmol malate, or 20 μmol lactate per 10 g fresh soil), 1000 ppm hydrogen, 0.2 and 2% oxygen or 10% carbon dioxide. Uptake kinetics for 100 ppm methane were followed periodically; uptake of atmospheric methane was measured after the soil was equilibrated at ambient air. Copper sulfate (0.01 nmol–10 μmol per g soil), nitrate (1 μmol per g soil) or a 10-fold concentrated mineral medium (0.1 ml per g soil); medium components as described by Widdel and Pfening [29] were added to soil for similar assays.

Various antibacterial antibiotics were added (2 ml to 10 g soil) as freshly prepared aqueous solutions: streptomycin (0.5 and 5 mg ml^{-1} = 0.1 and 1 mg gfw $soil^{-1}$) chloro tetracycline (0.5 and 5 mg ml^{-1} = 0.1 and 1 mg gfw $soil^{-1}$), chloramphenicol (0.5 mg ml^{-1} = 0.1 mg gfw $soil^{-1}$), and ampicillin 0.5 mg ml^{-1} = 0.1 mg gfw $soil^{-1}$). The anti-eukaryotic inhibitor cycloheximide (7.5 and 15 mg ml^{-1} = 3.75 and 7.5 mg gfw $soil^{-1}$) was also used. All assays were based on initial headspace methane concentration of 1.7 ppm for soil and of 100 ppm for culture experiments.

The response to methane starvation was assayed by incubating soils with sub-atmospheric methane levels. Glass jars with 10 g soil were flushed with synthetic air containing < 0.03 ppm methane. After 4 h and 2, 4, 8, 12, 16 and 38 days the jars were opened, allowed to equilibrate with ambient air and re-sealed for assays of atmospheric methane consumption rates.

Methane was analyzed by gas chromatography according to King and Adamsen [14]. For methane consumption above 100 ppm, rates were calculated from linear regressions of the time course data. Since methane consumption at lower concentrations was first order, oxidation rates were calculated using rate constants determined from exponential curve fits.

To determine patterns of carbon utilization, 15 μl of $^{14}CH_4$ (specific activity 2068 MBq $mmol^{-1}$, > 99% purity; Amersham Corp., Amersham, UK) was added to 10 g soil in 120-ml glass jars for assays at atmospheric methane concentration (3 kBq per assay). 100 μl $^{14}CH_4$ was used for assays with 170 ppm methane (20 kBq per assay). Total methane consumption was determined by gas chromatography. Jar headspace samples of 0.5 ml were collected for determination of $^{14}CO_2$ using a trap containing 3 ml of 0.5 M potassium hydroxide. A pulse of unlabeled methane was added to the soil after the initial methane depletion, and production of $^{14}CO_2$ was assayed for an additional period. Incorporation of radiolabel into phospholipids was assayed by extracting the soil at the termination of the incubation with methanol:dichloromethane according to Petersen et al. [22]. Radioactivity in the dichloromethane fraction was determined after evaporating the solvent, and resuspending the residue in liquid scintillation cocktail.

Cultures of *Methylobacter albus* and *Methylosinus trichosporium* OB3b were obtained from R.S. Hanson. Both cultures were grown in a phosphate-buffered mineral medium [29] to an optical density of 0.6 to 0.9 at a wavelength of 600 nm. Cultures were harvested by centrifugation and resuspended in medium to an optical density of 2.7. Two ml of the cell suspensions were placed in glass jars and incubated on a shaker with 120 rpm. Alternatively 2 ml of the cell suspensions were sprayed onto 10 g autoclaved sand or soil and transferred into jars incubated without shaking. Uptake rates of atmospheric methane were measured frequently over a time period of 15 days. After methane uptake was measured the jars were opened, covered with a wet towel to prevent desiccation and incubated at room temperature under ambient methane concentrations during intervals between uptake assays.

For traditional enrichment experiments in liquid media, 3 g fresh soil was added to 30 ml bicarbonate-buffered mineral medium [29] in 160-ml serum bottles. Potassium nitrate (0.4 g/l) was used as a nitrogen source. Trace element solution SL10a [30] was used with a 10-fold higher copper concentration. Vitamins were added as sterile solutions as described by Widdel and Pfennig [29]. Bottles were sealed with red rubber stoppers; the gas phases contained 10% CO_2 with various concentrations of methane. Enrichments were initiated with 100 ppm, 1000 ppm, or 1% methane in the gas phase, corresponding to about 3.2, 32 and 320 ppm (0.132, 1.32 and 13.2 μM) dissolved methane, respectively, at 20°C. Cultures

were isolated on agar or gelrite plates using standard plating and streaking techniques. Plates were incubated in anaerobic jars containing air with 10% CO_2 and approximately 5% methane.

3. Results

3.1. Response to enhanced methane availability

Methane oxidation rates for soil cores incubated continuously with atmospheric methane concentration were constant for 4 months (Fig. 1). A stable depth profile of methane developed within the soil cores (Fig. 2), but the specific activity for atmospheric methane consumption stayed homogeneous over depth. Uptake rates assayed with 17 ppm methane were 5–6 times higher than with 1.7 ppm, and did not change during 2 weeks of continuous incubation with 17 ppm methane (Fig. 1). Assayed with 170 ppm methane, rates were slightly higher than those measured with 17 ppm, and remained unchanged for 3 weeks of continuous incubation with 170 ppm methane. In addition also the rates for atmospheric methane consumption were unchanged after 3 weeks of 170 ppm incubation. Although

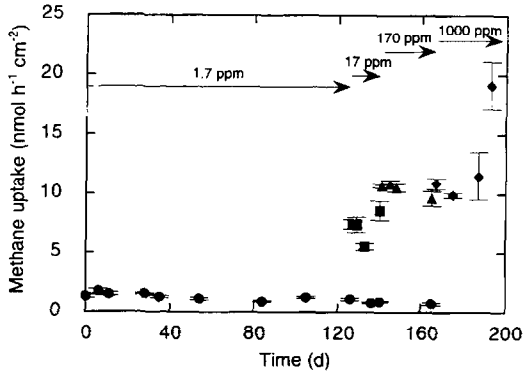

Fig. 1. Time course of methane uptake rates of soil cores filled exclusively with soil from the most active soil interval (4–6 cm). Soil cores were first incubated at atmospheric methane concentration (for 126 days) and later at 17 ppm (for 14 days), 170 ppm (for 24 days), and 1000 ppm methane (for 26 days). Methane consumption rates at atmospheric methane concentration (●), at 17 ppm (■), at 170 ppm (▲), and at 1000 ppm methane (◆) are shown over the time of the experiment. Values are means ±1 S.E. for triplicate assays.

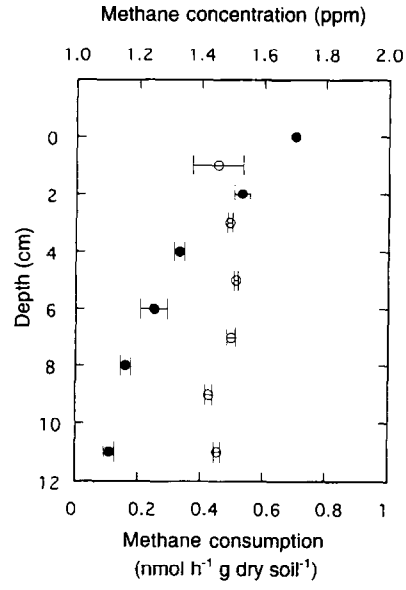

Fig. 2. Depth profile of methane concentrations (●) in the soil cores of the enrichment experiment and potential methane oxidation rates (○) over the core depth after 8 weeks of incubation at atmospheric methane concentration. Values are means ± 1 S.E. for triplicates.

methane consumption at 1000 ppm was initially similar to that at 170 ppm, rates increased slowly over a period of 5 weeks. As a result of the difficulty in equilibrating soil cores incubated at 1000 ppm with atmospheric methane, jar experiments (10 g soil in 120 ml jar) were used to compare activities at 1.7 and 1000 ppm methane during an incubation with 1000 ppm methane. After 5 weeks of incubation at 1000 ppm methane, the oxidation rate at 1000 ppm methane increased 176-fold compared to the initial rate, the rate for atmospheric methane oxidation however increased only 5.5-fold (Fig. 3). Both rates decreased rapidly after incubation at atmospheric methane concentration.

The methane consumption rate at 170 ppm methane was 90.7 nmol gfw $soil^{-1}$ d^{-1}, 36-fold higher than the rate at atmospheric methane concentrations (2.5 nmol gfw $soil^{-1}$ d^{-1}). However, the fractions of methane ($^{14}CH_4$) oxidized and recovered as $^{14}CO_2$ were very similar: 51.9% at atmospheric methane concentration and 46.6% at 170 ppm methane (Fig. 4). The label recovered from soil after phospholipid extraction was 0.35% at atmo-

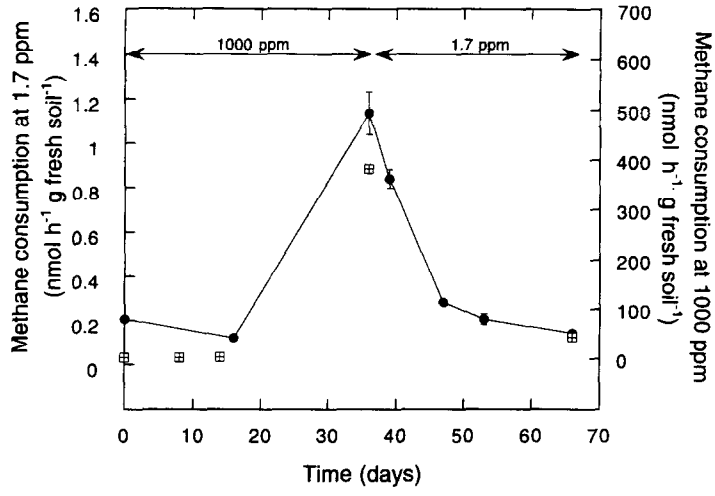

Fig. 3. Rates of methane consumption were measured at methane concentrations of 1.7 ppm (●) or 1000 ppm (crossed square). Soils were incubated with 1000 ppm methane in the headspace for the first 36 days; then headspace concentrations were reduced to 1.7 ppm methane for the subsequent 30 days. Headspace concentrations were adjusted as necessary for assays of uptake rates at 1.7 or 1000 ppm; after the assay, head spaces were re-established at the designed concentration for incubation. Values are means ±1 S.E. for quadruplicate assays.

spheric methane concentration, and 0.22% at 200 ppm methane.

3.2. Response to multi-carbon substrates and nutrients

None of the substrates tested (Table 1) stimulated methane consumption at atmospheric methane concentration or 100 ppm methane. The rates for methane consumption at both concentrations did not change over a period of 3–8 weeks of incubation with 100 ppm methane. Addition of copper (0.01 nmol, 1 nmol, 0.1 μmol, 1 μmol or 10 μmol per g soil) did not affect atmospheric methane uptake. Low oxygen concentration (0.2 or 2%) inhibited methane consumption, while elevated carbon dioxide concentrations (10%) had no effect. Addition of mineral medium showed a strong, whilst nitrate showed a slight inhibitory effect.

3.3. Response to methane starvation

Forest soil incubated with synthetic air containing < 0.03 ppm methane lost its capacity for methane

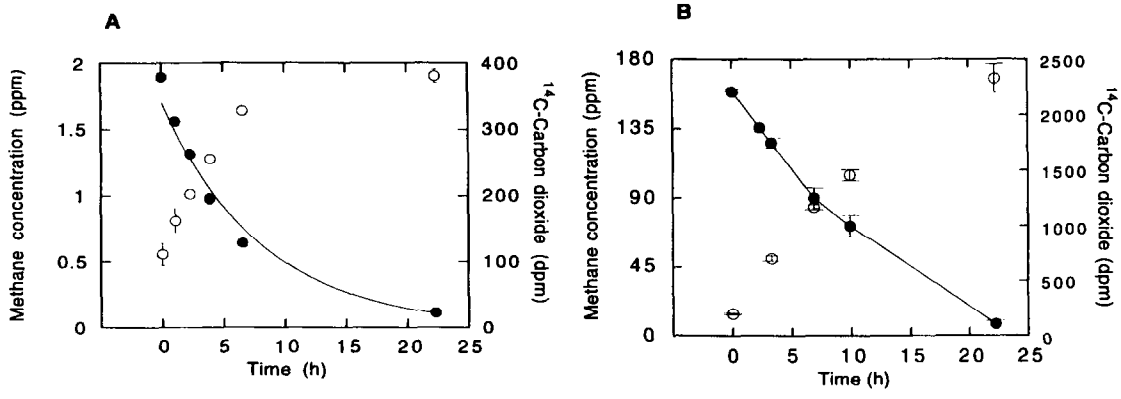

Fig. 4. Methane consumption (●) and carbon dioxide production (○) with soil at atmospheric (**A**) and 170 ppm methane concentration (**B**). Data present means of quintuples; bars indicate ±1 S.E.

Table 1
Response of soil methane consumption to substrate addition. Relative methane uptakes at 1.7 and 100 ppm methane are given in % of the water control assay

Substrate added (concentration gfw soil^{-1})	Relative uptake at 1.7 ppm [a]	Relative uptake at 100 ppm [b]
Control	100	100
Glucose (1 μmol)	101.4	101
Starch (0.2 mg)	–	99.5
Yeast extract (0.2 mg)	–	66.5
Methanol (1 μmol)	98.6	28.8
Ethanol (2 μmol)	–	84.2
Formate (2 μmol)	102.8	–
Acetate (2 μmol)	–	40.4
β-Hydroxybutyrate (1 μmol)	92.0	–
Lactate (2 μmol)	–	73.5
Malate (2.5 μmol)	103.3	–
Hydrogen (100 ppm) [c]	–	86.5
Oxygen (0.2%) [c]	–	6.9
Oxygen (2%) [c]	–	3.7
Carbon dioxide (10%) [c]	99.3	95.6
Nitrate (1 μmol)	88.3	–
Medium (0.1 ml)	53.3	–

– = not determined.
[a] Samples were incubated at 1.7 ppm methane.
[b] Samples were incubated at 100 ppm methane.
[c] Gas phase concentrations.

consumption exponentially over time (Fig. 5). After 6.3 days of incubation only 50% of the original activity remained, and after 38 days, activity was 14% of the initial uptake (10.5 nmol gfw soil^{-1} d^{-1}). Activity could not be restored after re-introduction of atmospheric methane, e.g. 47.5% of the

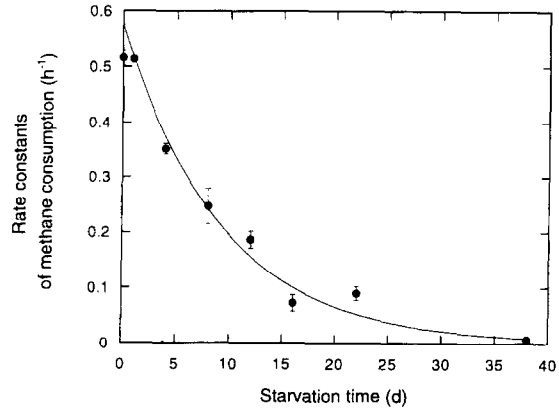

Fig. 5. Rate constants of atmospheric methane consumption by soil incubated under hydrocarbon free air for various times between 4 h to 38 days. After the incubation time, soil was re-exposed to ambient air, and methane uptake rates were measured. Values are means ±1 S.E. for quadruplicate assays.

original activity remained after incubation with synthetic air for 8 days; this level of activity did not increase after subsequent incubation with ambient methane concentrations for 16 days.

3.4. Response to antibiotic addition

Atmospheric methane consumption by soil was inhibited by the addition of antibacterial antibiotics (streptomycin, chloro tetracycline, chloramphenicol, ampicillin) as well as an eukaryotic antibiotic (cycloheximide). Streptomycin at a concentration of 0.1 mg g soil^{-1} only slightly depressed methane

Table 2
Rate constants (h^{-1}) for atmospheric methane uptakes by cell suspensions of *Methylosinus trichosporium* OB3b and *Methylobacter albus*. Two ml of the cultures (OD_{600} 2.7) were incubated in 120-ml glass jars on a shaker, or were sprayed on 10 g autoclaved soil or sand. Rate constants (h^{-1}) for 10 g fresh are given for comparison. Values are means of triplicates ±1 S.E.

Culture and condition	Time (days)			
	0	2	6	15
M. albus	0.0822 ± 0.0139	0.0612 ± 0.0010	0.003 ± 0.001	0.0018 ± 0.0004
M. albus on sand	0.1908 ± 0.0082	0.2748 ± 0.0204	0.0234 ± 0.001	0.0048 ± 0.0003
M. albus on soil	–	–	–	–
M. trichosporium OB3b	0.1002 ± 0.0099	0.084 ± 0.0032	0.0516 ± 0.0007	0.0114 ± 0.0008
M. trichosporium OB3b on sand	0.2604 ± 0.0042	0.2856 ± 0.0014	0.036 ± 0.0031	0.0168 ± 0.0011
M. trichosporium OB3b on soil	–	–	–	–
Soil from the 4–6 cm interval	0.1854 ± 0.0072	0.2356 ± 0.0091	0.2118 ± 0.0022	0.2754 ± 0.0011

– = not detectable.

uptake (92% of control uptake rates); however, 1 mg g soil^{-1} streptomycin substantially inhibited methane consumption (65% of the control methane uptake). With chloro tetracycline methane consumption was 57% and 19% of the original activity at 0.1 mg g soil^{-1} and 1 mg g soil^{-1}, respectively. Chloramphenicol (0.1 mg g soil^{-1}) depressed methane consumption by 40%. Ampicillin at 0.1 mg g soil^{-1} depressed methane uptake by 73% of the control activity and by 46% at 0.5 mg g soil^{-1}. At a concentration of 1.5 mg g soil^{-1} cycloheximide methane consumption was not affected, however, uptake was only 77% of the control activity at 3.0 mg g soil^{-1}. Cell suspensions of *M. trichosporium* OB3b were similarly sensitive to both the antibacterial antibiotics, and cycloheximide.

3.5. Atmospheric methane consumption by Methylosinus trichosporium OB3b and Methylobacter albus

Dense cell suspensions ($9.5 \cdot 10^8$ cells/ml) of *M. trichosporium* and *M. albus* consumed atmospheric methane with rate constants of 0.10 h^{-1} assay (2 ml culture)$^{-1}$ and 0.0822 h^{-1} assay (2 ml culture)$^{-1}$, respectively (Table 2). The rate constant for forest soil from the 4–6 cm depth interval was approximately twice as high (0.186 h^{-1} assay (10 g soil)$^{-1}$). Culture cell suspensions sprayed on autoclaved soil with a pH 4.5 did not consume atmospheric methane. However, cell suspensions sprayed on washed and autoclaved sand showed 2.3 to 2.6 times greater methane consumption rates than cell suspensions (of the same optical density in the same volume of medium) incubated without a surface support. Controls of autoclaved sand did not consume any methane. The capacity of cultures to consume atmospheric methane was initially higher in cell suspensions sprayed on sand: activities were unchanged in the sand cultures after 2 days, whereas methane uptake rates for the control cultures decreased by 16–26%. After 6 days, *M. albus* on sand still showed 13% of the original activity, while the control culture in liquid only showed 4%. In contrast, cell suspensions of *M. trichosporium* OB3b retained 52% of the initial activity, while cultures on sand retained only 14% of their original methane oxidizing capacity. After 15 days, the activities of all cultures decreased to 2–12% of the original consumption rates.

3.6. Enrichment experiments in mineral medium

Enrichment cultures using soil from the 4–6 cm depth interval, a bicarbonate-buffered medium and gas phase methane concentrations of 100 ppm showed no methane consumption over 12 months. However, in enrichments with 1000 ppm methane, methane was depleted after 4 weeks at 28°C and after 3 months at 10°C. Methane consumption was stimulated by repeated addition of 1000 ppm methane. Four pure cultures were isolated at 10°C and 28°C on agar or gelrite plates. All isolates consumed methane on plates or slants but did not grow in liquid culture.

4. Discussion

Methane consumption by soil cores filled with the most active soil layer (4–6 cm depth) and incubated with ambient methane concentration remained stable for more than 4 months (Fig. 1). Even though the surface layer of the cores was exposed to approximately twice the in situ methane concentration, the rates of methane consumption did not increase within 18 weeks of incubation (Fig. 2). The soil cores developed a stable methane profile within a soil depth of 12 cm reflecting a steady state of diffusion and methane consumption. After incubation for 2 weeks at 17 ppm, the amount of carbon metabolized was equivalent to the amount of carbon metabolized at 1.7 ppm within 10–12 weeks. Similarly methane uptake at 170 ppm was 8 times that at 1.7 ppm, and the amount of carbon metabolized during 3 weeks of incubation was equivalent to 24–27 weeks of atmospheric methane consumption. However, methane uptake rates remained constant during the 17 and 170 ppm incubation periods suggesting that the soil methanotrophs were unable to grow in response to increased methane availability.

The ratio of carbon dioxide produced to methane metabolized indicates the potential for carbon incorporation into cell mass. For methanotrophs in culture, approximately 50% of the methane consumed is dissimilated while the rest is assimilated [2,12]. From previous work it has been evident that soil methanotrophs have a limited capacity to grow when using atmospheric methane [16,24]. One reason could be that at low substrate concentrations the energy de-

mands for maintenance may require a high level of methane dissimilation, leaving only a small amount of carbon for cell mass syntheses. The percentage of $^{14}CO_2$ produced from $^{14}CH_4$ both at 1.7 and 170 ppm was very similar (51.9% at 1.7 ppm and 46.6% at 170 ppm, Fig. 4) and comparable to data for cultures grown with high methane concentrations. The amount of label recovered in phospholipids as an indicator for soil biomass was also similar at 1.7 and 170 ppm methane, 0.35% and 0.22%, respectively. Although isotopic dilution may cause underestimates of the true level of dissimilation [15], the 'pulse-chase' results suggest that such problems were minimal. Thus, the energetic demands for soil methanotrophs using atmospheric methane may be high relative to those for typical culture conditions. Alternatively, the demands of cell maintenance for carbon (e.g. membrane, protein, nucleic acid) turnover may result in an apparently normal level of dissimilation, but preclude growth until cell-specific methane uptake rates exceed some threshold value, probably requiring gas phase methane concentrations > 200ppm.

The capacity for soil methane consumption was stimulated after incubation at 1000 ppm methane, probably as a result of methanotrophic growth. However this increased activity could not be maintained during subsequent incubation with atmospheric methane concentrations (Fig. 3). It is likely that incubation with 1000 ppm methane enriched for methanotrophic bacteria with high K_m and high V_{max} values, characteristics that are atypical for atmospheric methane consumption by soil [4]. The loss of methanotrophic activity at ambient methane concentration is consistent with results of Roslev and King [23], who showed that such levels elicited a starvation response from cultures of *Methylosinus trichosporium* OB3b.

Soil incubated with low methane (< 0.03 ppm) air lost the capacity for atmospheric consumption exponentially (Fig. 5). The methane-oxidizing activity of soil starved for 8 days was 47.5% of the original activity; no recovery was evident after 16 days of incubation at ambient methane concentration. These results suggest that atmospheric methane is the primary substrate supporting the activity of soil methanotrophs. Since eight days of starvation decreased the activity irreversibly, it appears that atmospheric methane concentration is basically sufficient for maintaining methanotrophic populations in soils in a steady-state with respect to losses such as cell death and grazing by protozoa. The sensitivity of the methane-consuming activity in soil to several antibiotics that inhibit protein synthesis indicates a role for protein turnover in methane oxidation within 3–4 h, since the inhibitory effect of the antibiotics appears within that time.

Methanotrophic activity did not appear to respond to a variety of possible co-substrates, including C_1 substrates (methanol, formate) and various complex organics (glucose, starch, yeast extract; Table 1). Hydrogen also did not influence soil methane consumption. Co-metabolism of organic compounds or hydrogen, if it occurred, could enhance growth or maintenance for cells exposed to ambient or sub-ambient levels of methane. Wolf and Hanson [31] described the isolation of acid-tolerant (pH 4) methane-oxidizing yeasts from soils that also grew on complex organic substrates. A facultative methanotrophic bacterium was described by Patt et al. [21] that incorporated ^{14}C-carbon into cell mass from growth on $^{14}CH_4$ (in the presence of unlabeled glucose). The absence of any response in our experiments indicates that soil methanotrophs may have a more limited substrate spectrum than has been described for methanotrophs in culture [3,8,9,28].

The soil from the 4–6 cm depth interval used in this study had a low extractable nitrogen content (6.5 μmol ammonium gdw soil^{-1}, 0.3 μmol nitrate gdw soil^{-1}; [24]) that might be growth limiting for methanotrophs. However, the lack of any stimulation of methane consumption by nitrate additions suggests that assimilable nitrogen is not a major limiting factor. In contrast the response of these soils to ammonium was inhibitory, as described elsewhere [16,24]. Copper availability also did not appear to limit growth or activity, since addition of copper sulfate did not affect methane consumption, even at relativ high concentrations (10 μmol gfw soil^{-1}). In a more general sense, the increased availability of various cations and anions was not limiting for growth or enzyme activity, since addition of a mineral medium did not enhance methane consumption over a period of 8 weeks. In contrast lower oxygen concentrations (0.2 and 2%) in the headspace partially inhibited methane consumption indicating that

soil methane consumption may not be due to microaerophiles.

Dense cell suspensions of *Methylosinus trichosporium* OB3b and *Methylobacter albus* consumed atmospheric methane. However, assays with 2 ml culture suspensions of OD_{600} 2.7 ($= 1.91\ 10^9$ cells) showed half the rate constant of 10 g forest soil (Table 2). Thus the activity of 1 g forest soil was equivalent to $3.8 \cdot 10^8$ cells of the cultures, which seems unrealistically high for soil methanotrophs. A lower number of methanotrophs with different kinetic parameters than the cultures is much more likely, as Bender and Conrad [3] pointed out. Autoclaved sand enhanced methane consumption by the cultures, probably in part because of enhanced diffusive transport of methane. However, it was still evident that the cultures were unable to sustain activity at atmospheric methane. This suggests that existing methanotrophic isolates may share many characteristics with soil methanotrophs, yet differ fundamentally in kinetic properties. Surface matrix of soil could have an important impact on the methane consuming activity in soil. The lack of surface matrix could be a reason for our inability to grow our soil isolates of methanotrophs in liquid culture.

In conclusion, our results provide evidence that methane is the primary substrate (carbon and energy source) of soil methanotrophs. The methane consuming activity of soil was stimulated at methane concentrations > 1000 ppm, but not by organic substrates or inorganic co-factors. Soil incubated under hydrocarbon-free air (< 0.03 ppm methane) lost the capacity for methane consumption exponentially, and did not recover with subsequent incubation at ambient methane concentration. The increased activity of soils incubated with 1000 ppm methane was much greater for rates measured at 1000 ppm methane than those measured at atmospheric methane, indicating that the population enriched by 1000 ppm methane had different kinetic characteristics than the methanotrophs responsible for consuming ambient methane. The fraction of methane carbon oxidized was the same at atmospheric methane concentrations and at 170 ppm methane, and generally similar to reports for pure cultures of methanotrophs incubated with percent levels of methane. Although the high-affinity soil methanotrophs remain uncultured, our results indicate that their substrate utilization patterns are typical of known methanotrophs, but with different kinetics for methane uptake.

Acknowledgements

We thank Peter Roslev and Aram Calhoun for many helpful discussions. This work was supported by NASA NAGW-3746, NSF DEB 91-07315, and a fellowship of the Deutsche Forschungsgemeinschaft (S.S.).

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